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Protection against Anthrax with Recombinant Virus-Expressed Protective Antigen in Experimental Animals

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We previously described the cloning and expression of the protective antigen (PA) gene of *Bacillus anthracis* in both vaccinia virus and a baculovirus. The antigenicity of the PA products was characterized. PA expressed by the recombinant vaccinia viruses elicited a partial protective immune response against a lethal *B. anthracis* spore challenge in guinea pigs and mice. The WR strain vaccinia virus recombinant (WR-PA) protected 60% of male mice and 50% of guinea pigs. WR-PA elicited high anti-PA antibody titers in mice but not in guinea pigs. Connaught strain vaccinia virus recombinants failed to protect any immunized animals. PA purified from baculovirus recombinant-infected cultures plus adjuvant partially protected male CBA/J mice and completely protected female Hartley guinea pigs from challenge. Both the recombinant and nonrecombinant PA preparations combined with adjuvant elicited high anti-PA antibody titers in Hartley guinea pigs and CBA/J mice. These data demonstrate that the recombinant baculovirus- and vaccinia virus-produced PAs were immunogenic in both guinea pigs and mice, that the baculovirus-PA recombinant was a useful source of immunogenic PA, and that vaccinia virus-PA recombinants may be feasible live anthrax vaccine candidates worthy of consideration for further development as live vaccines.

A primary goal of recent research on anthrax has been the development of prototype anthrax vaccine candidates (6–9, 29). Currently, livestock are vaccinated with spores of the live toxigenic, nonencapsulated Sterne strain of *Bacillus anthracis* (24), which occasionally causes necrosis at the injection site but seldom causes death. The primary immunogenic component of the human vaccine is anthrax protective antigen (PA), prepared by the Michigan Department of Public Health (MDPH-PA). This vaccine consists of aluminum hydroxide-adsorbed supernatants from fermenter cultures of a toxigenic, nonencapsulated strain of *B. anthracis*, V770-NP1-R (21). Immunization with MDPH-PA can induce local pain, edema, and erythema (1), and frequent boosters are required. There is evidence that in guinea pigs some virulent strains of *B. anthracis* are partially refractive to immunization with MDPH-PA (11, 27). In addition, MDPH-PA fails to protect mice against *B. anthracis* (28, 29).

We recently reported the construction of recombinants of *Autographa californica* nuclear polyhedrosis virus (baculovirus) and vaccinia virus that contain the PA gene (3). Baculovirus recombinants can express foreign genes to high levels when the foreign gene is inserted into the polyhedrin gene of baculovirus (13, 16–18, 22, 23). Vaccinia virus has been developed as a live infectious expression vector for foreign genes inserted into the vaccinia virus thymidine kinase gene (14, 15, 19, 20). The virus recombinants produced a PA product antigenically similar to the PA produced by the Sterne strain of *B. anthracis* (3). When mice were immunized with baculovirus-PA recombinant (Bac-PA)-infected cells or infected with live vaccinia virus-PA recombinants, a high anti-PA titer was elicited, as measured by an antibody capture enzyme-linked immunosorbent assay (ELISA; 3).

The objective of these studies was to evaluate the protective efficacy of vaccinia virus-PA recombinants and PA produced by Bac-PA as vaccines against lethal anthrax infection of laboratory rodents. CBA/J male mice and female Hartley guinea pigs were immunized with either live vaccinia virus-PA recombinants or PA produced and purified from SF-9 cells (*Spodoptera frugiperda*) infected with the recombinant virus Bac-PA. The Hartley guinea pig was established previously as a good animal model for testing the protective immunogenicity of anthrax vaccines (5, 7, 11). The CBA/J mouse model has also been developed for the study of susceptibility to anthrax and for testing the protective efficacy of anthrax vaccines (28–31). *B. anthracis* Ames spores were used for parenteral challenge of immunized animals. Challenge with *B. anthracis* Ames is the most stringent test of vaccine efficacy, because it is the most difficult *B. anthracis* strain to immunize against with MDPH-PA and other vaccines (11, 27, 28).

Our studies provided data that suggest that a vaccinia virus-PA recombinant and the PA produced from Bac-PA are feasible *B. anthracis* vaccine candidates.

MATERIALS AND METHODS

Viruses and cells. The WR strain of vaccinia virus (a mouse-virulent, neurotropic strain) and a less virulent vaccinia virus strain, Connaught, were used to make vaccinia virus-PA recombinants (3). The Connaught strain is a human vaccine strain of vaccinia virus which originated from the New York City Board of Health strain. Vaccinia virus strain WR, strain Connaught, and vaccinia virus recombinants Connaught-PA (Con-PA) and WR-PA were prepared, propagated, and assayed in Vero cells, as described previously (2, 3, 15). Bac-PA was prepared, propagated, and assayed in SF-9 cells, as described previously (3, 25). The virus-PA recombinants were prepared with identical PA gene clones. Briefly, a phagemid vector that contained the PA gene, pBLSCRPPA, was kindly supplied by J. Lowe, U.S. Army Medical Research Institute of Infectious Diseases (USAM-

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RIID). The PA gene was subcloned into the above vector (12a) from the plasmid pPA26 (32).

Protective antigen. Purified *B. anthracis* Sterne PA (Sterne-PA) was provided by S. H. Leppla (Bacteriology Division, USAMRIID) (10). PA produced by Bac-PA will be referred to as baculovirus PA. PA that was produced by the recombinant virus Bac-PA in infected SF-9 cells was purified by immunoaffinity column chromatography. The mouse monoclonal antibody 3B6 (12) was conjugated to hydrazide resin from BioRad Inc., Rockville Center, N.Y. (19a). Briefly, SF-9 cells were infected with Bac-PA at a multiplicity of infection of 10 PFU per cell. At 50% cell death, cells were harvested and lysed, and the soluble cell lysate material was passed through an immunoaffinity column. Specifically bound PA was eluted and either assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie blue or analyzed by Western blot (immunoblot) with rabbit anti-PA antibody serum.

Experimental animals. Female Hartley guinea pigs, 350 to 375 g, were purchased from Charles River Laboratories, Wilmington, Mass. Male 6- to 10-week-old CBA/J mice were obtained from Jackson Laboratories, Bar Harbor, Me.

Adjuvant. A mixture, which we have termed "TriMix," of monophosphoryl lipid A (MPL), trehalose dimycolate (TDM) from *Mycobacterium phlei*, and the delipidated, deproteinated cell wall skeleton (CWS) from *M. phlei* was purchased from Ribi Immunochem Research, Inc., Hamilton, Mont.

***B. anthracis* challenge spores.** Spores from the vaccine-resistant *B. anthracis* Ames strain were produced, harvested, purified, and stored according to previously published methods (9). Guinea pigs were challenged intramuscularly with 4,000 spores in 0.2 ml of Dulbecco phosphate-buffered saline (PBS) containing 0.1% gelatin. Mice were challenged subcutaneously (s.c.) with 350 spores in 0.2 ml of PBS containing 0.1% gelatin. The 50% lethal dose (LD_{50}) of Ames spores for guinea pigs is 100 (9), and for female CBA/J mice it is 35 (28).

Immunization and challenge of guinea pigs. Guinea pigs were immunized at 0 and 4 weeks, then challenged at 8 weeks with 4,000 (40 LD_{50}s) *B. anthracis* Ames spores. Immunization groups were as follows. (i) Control guinea pigs were given 0.5 ml of PBS intramuscularly at 0 and 4 weeks. (ii) Guinea pigs received 0.5 ml of a solution containing Sterne-PA (70 μg) and TriMix (25 μg of MPL, 125 μg of TDM, 125 μg of CWS), with 0.2 ml s.c. in each of two sites and 0.1 ml intraperitoneally (i.p.) at 0 weeks. At 4 weeks, the guinea pigs were boosted with 12.5 μg of PA in TriMix and injected as described above. (iii) At 0 weeks, guinea pigs received 0.5 ml of baculovirus PA (70 μg) and TriMix as described above (0.2 ml s.c. in each of two sites and 0.1 ml i.p.). The animals were boosted at 4 weeks with 12.5 μg of baculovirus PA and TriMix as described above. (iv) At 0 weeks, shaved guinea pigs were immunized with WR-PA (2×10^{10} PFU/ml) by scarifying four sites with 50 μl per site. Guinea pigs were boosted at 4 weeks with 0.2 ml i.p. and 0.1 ml intradermally. (v) At 0 weeks, shaved guinea pigs were immunized with Con-PA (10^9 PFU/ml) by scarifying four sites with 50 μl per site. At 4 weeks, the guinea pigs received 0.2 ml i.p. and 0.1 ml intradermally.

Immunization and challenge of mice. CBA/J mice were immunized as described below and challenged at 8 weeks with 350 (10 LD_{50}s) *B. anthracis* Ames spores. TriMix adjuvant consisted of 10 μg of MPL, 40 μg of TDM, and 40 μg of CWS. Immunization groups were as follows. (i) Control mice were given 0.2 ml of PBS s.c. at 0, 2, and 4 weeks. (ii) Mice received 0.2 ml of a solution containing Sterne-PA (28 μg) s.c. with TriMix at 0, 2, and 4 weeks. (iii) Mice received 0.2 ml of a solution containing Sterne-PA (28 μg) s.c. without TriMix at 0, 2, and 4 weeks. (iv) Mice received 0.2 ml of a solution containing baculovirus PA (28 μg) s.c. with TriMix at 0, 2, and 4 weeks. (v) Mice were immunized with the live WR virus (approximately 9×10^9 PFU per dose) by tail scarification. Six weeks later, mice received 0.14 ml of WR virus (4.2×10^{10} PFU per dose) i.p. (vi) Mice were immunized with WR-PA (approximately 3×10^9 PFU per dose) by tail scarification. Six weeks later, mice received 0.14 ml of WR-PA (1.4×10^{10} PFU per dose) i.p.

Serology. Two days before challenge, guinea pigs and mice were bled by puncture of the heart or retro-orbital sinus, respectively, and their sera were assayed for antibody to Sterne-PA by ELISA (11, 29). The ELISAs were performed by using a modification of the assay described by Little and Knudson (11). Pooled sera from unimmunized animals and an anti-PA monoclonal antibody were included in each assay as negative and positive controls, respectively. Briefly, 100 ng of purified PA (10) diluted in 0.05 M sodium borate buffer (pH 9.5) was added to each well of 96-well microtiter plates in a 100-μl volume. Plates were incubated at 4°C overnight, blocked with PBS–0.5% gelatin, and washed with PBS–0.05% Tween 20, and 100-μl sample volumes were added to each well. Serum samples were appropriately diluted in PBS–0.5% gelatin–0.05% Tween 20. Plates were incubated for 2 h at 37°C, washed as described above, and then incubated with goat antibody to mouse immunoglobulins G, A, and M (Kirkegaard and Perry) conjugated to horseradish peroxidase for 2 h at room temperature. Plates were washed as described above and incubated at room temperature with 2,2′-azino-bis(3-ethylbenzthiazolinesulfonic acid) (Sigma) at 1 mg/ml in 0.1 M sodium citrate buffer (pH 4.0)–0.003% hydrogen peroxide. Plates were read at A_{405} (11). Serum samples from all immunized animals (guinea pigs and mice) were tested. ELISA titers of individual serum samples were the reciprocal of the highest dilution yielding an absorbance which exceeded the negative control absorbance by at least 0.2 U. The average ELISA titer of each group was the geometric mean of the individual titers.

RESULTS AND DISCUSSION

Purified Sterne-PA from the Sterne strain, an attenuated, toxigenic, nonencapsulated strain (24) of *B. anthracis* (10), was shown previously to elicit a protective immune response in Hartley guinea pigs (4) and in female CBA/J mice (28) against *B. anthracis* Ames spore challenge. We used these established animal protection models to evaluate the efficacy of three virus recombinant *B. anthracis* vaccine candidates (WR-PA, Con-PA, and baculovirus PA) against Sterne-PA to protect CBA/J mice and female Hartley guinea pigs against a lethal *B. anthracis* Ames spore challenge. Sterne-PA was used as a recognized purified PA preparation in these protection studies for evaluating the relative immunogenic and protective properties of the virus recombinant PA candidate vaccines.

In previous experiments, Sterne-PA protected 89% of female CBA/J mice against a lethal Ames spore challenge (28). In the present experiment, male CBA/J mice were used because females were unavailable for an extended period of time from the vendor; the same vendor has been used as the source of mice for all our studies with the anthrax mouse model (28–30). Sterne-PA, with or without adjuvant, failed to significantly protect the male CBA/J mice from challenge,

TABLE 1. Protection of mice against *B. anthracis* Ames with recombinant vaccinia virus and purified recombinant and nonrecombinant PAs

Vaccine[a]	Anti-PA titer (SD)[b]	Response to challenge		
		No. of survivors/ total (%)[c]	TTD (days) (SD)[d]	*P* value[e]
WR-PA	263,027 (2.4)	15/25 (60.0)	7.5 (1.4)	0.0016
WR	10	1/12 (8.3)	4.6 (1.2)	1.0000
Baculovirus PA + TriMix	199,526 (2.6)	5/10 (50.0)	5.1 (1.5)	0.0325
Sterne-PA	16,144 (2.8)	1/12 (8.3)	5.0 (1.2)	1.0000
Sterne-PA + TriMix	287,276 (1.8)	3/11 (27.3)	6.5 (1.3)	0.2140
PBS	<10	0/10 (0)	3.8 (1.2)	

[a] Mice were immunized with PBS or with the WR strain of vaccinia virus (controls) or with the vaccine preparations described in the text. These preparations included WR-PA, Sterne-PA, and baculovirus PA.

[b] Serum anti-PA antibody titers were determined by ELISA in sera collected from all mice 2 days before challenge. Titers are shown as the geometric mean reciprocal. SD, standard deviation from the geometric mean.

[c] Immunized mice were challenged with *B. anthracis* Ames as described in the text. Data are shown as the number of survivors of the total number challenged, with the percent survival in parentheses. Survivors refer to animals still alive 14 days after challenge.

[d] TTD, geometric mean time to death in days; SD, standard deviation from the geometric mean.

[e] Percent survival of the PBS control versus that of the vaccine candidates was tested with the two-tailed Fisher's exact test at a 0.007 significance level and a Bonferroni correction for multiple comparisons. Percent survival between selected mouse groups was also tested, with the two-tailed Fisher's exact test at a 0.007 significance level and a Bonferroni correction. When results with WR-PA and Sterne-PA plus TriMix were compared, *P* was 0.1460; when results with baculovirus PA plus TriMix and Sterne-PA plus TriMix were compared, *P* was 0.3870. The statistical analysis program used was the SAS version 6.04 IBM/PC.

with 27.3 and 8.3% survival, respectively (Table 1). However, animals immunized with Sterne-PA and TriMix had high anti-PA titers (Table 1). Therefore, as has been reported previously, high anti-PA titers do not necessarily provide protection from challenge (Table 1) (9, 26–29). Sterne-PA combined with adjuvant did provide complete protection against a lethal Ames spore challenge in female Hartley guinea pigs (Table 2). The anti-PA antibody titers of animals in this group were also high compared with those of the PBS control (Table 2).

Baculovirus PA, in combination with adjuvant, elicited high anti-PA titers similar to those elicited by Sterne-PA with adjuvant in mice and guinea pigs (Tables 1 and 2). Baculovirus PA protected 50% of the mice (Table 1), but protection was not significantly different from that observed with Sterne-PA plus TriMix (27.3%) (Table 1). Thus, baculovirus PA appears to be comparable to Sterne-PA as a protective immunogen in CBA/J mice. This observation is supported by the fact that baculovirus PA and Sterne-PA proteins were antigenically indistinguishable (3). Baculovirus PA, combined with adjuvant, provided complete protection in guinea pigs against a lethal Ames spore challenge. The elicited immune response and the protective efficacy of the baculovirus PA experimental vaccine in guinea pigs were nearly identical to those observed in animals immunized with Sterne-PA (Table 2). These data demonstrate not only that the baculovirus PA was immunogenic in guinea pigs but that the immune response was completely protective.

TABLE 2. Protection of guinea pigs against *B. anthracis* Ames with recombinant vaccinia virus and purified recombinant and nonrecombinant PAs

Vaccine[a]	Anti-PA titer (SD)[b]	Response to challenge		
		No. of survivors/ total (%)[c]	TTD (days) (SD)[d]	*P* value[e]
PBS	8 (2.1)	0/7 (0)	4.1 (1.9)	
Con-PA	11 (4.6)	1/8 (12.5)	2.9 (1.3)	1.0000
WR-PA	237 (4.0)	4/8 (50.0)	4.7 (1.6)	0.0770
Baculovirus PA + TriMix	51,794 (2.3)	8/8 (100)		0.0002
Sterne-PA + TriMix	60,097 (5.3)	8/8 (100)		0.0002

[a] Guinea pigs were immunized at 0 and 4 weeks as described in the text.

[b] Reciprocal geometric mean anti-PA ELISA titers were determined from sera from guinea pigs bled 8 weeks after the first immunization. SD, standard deviation from the geometric mean.

[c] Animals were challenged at 8 weeks with *B. anthracis* Ames as described in the text. Data are shown as the number of survivors of the total number challenged, with the percent survival in parentheses. Survivors refer to animals still alive 14 days after challenge.

[d] TTD, geometric mean time to death in days; SD, standard deviation from the geometric mean.

[e] Percent survival of the PBS control versus that of the vaccine candidates was tested, with the two-tailed Fisher's exact test at a 0.008 significance level and a Bonferroni correction for multiple comparisons. Percent survival between selected guinea pig groups was also tested, with the two-tailed Fisher's exact test at a 0.008 significance and a Bonferroni correction. When results with WR-PA and Sterne-PA plus TriMix were compared, *P* was 0.0770; when results with WR-PA and baculovirus PA plus TriMix were compared, *P* was also 0.0770. The statistical analysis program used was the SAS version 6.04 IBM/PC.

Vaccinia virus-PA recombinants are capable of replicating and expressing PA upon inoculation into animals (3). Male CBA/J mice vaccinated with the live vaccinia virus recombinant WR-PA generated uniformly high anti-PA-specific antibody. The mice were only partially protected (60%) against an Ames spore challenge (Table 1), as shown previously for three other live vaccines, the Sterne strain veterinary vaccine and the *B. subtilis* PA-producing recombinant strains PA2 and PA7 (28, 29).

Guinea pigs immunized with the live vaccinia virus-PA recombinant, WR-PA, were partially protected (50%) from an Ames spore challenge (Table 2). WR-PA elicited lower anti-PA titers in guinea pigs than did the baculovirus PA and Sterne-PA, and protection provided by the WR-PA vaccine was not different from that provided by the PBS control (Table 2). These studies demonstrate that of the three vaccines tested, baculovirus PA and Sterne-PA provided the best protection to female Hartley guinea pigs against a lethal Ames spore challenge (Table 2). The mean time to death in the WR-PA-immunized guinea pig group was not altered by vaccination.

We found that female CBA/J mice immunized with Con-PA were not protected significantly against a lethal Ames spore challenge (data not shown). Similarly, in this study, guinea pigs immunized with the live Con-PA recom-

binant were not significantly protected (Table 2). Con-PA did not elicit an anti-PA titer that differed significantly from the PBS control group. It is not clear why WR-PA but not Con-PA provided partial protection in mice and guinea pigs. However, the specific host response to the two different strains of recombinant vaccinia virus is likely a primary factor, since both WR-PA and Con-PA were shown previously to produce approximately 0.2 pg of PA per infected Vero cell by ELISA (3). It is possible that the Con-PA recombinant failed to replicate in the immunized animals to the same extent as the WR-PA recombinant. Thus, WR-PA could have expressed larger quantities of recombinant PA over a longer period of time in the inoculated animal host than Con-PA. Both Con-PA- and WR-PA-immunized guinea pigs developed lesions at the vaccine scarification sites (data not shown), suggesting initial vaccinia virus recombinant replication at the site of inoculation.

The *B. anthracis* vaccine licensed for human use in the United States (MDPH-PA) does not protect CBA/J mice against an Ames spore challenge (28). Guinea pigs immunized with MDPH-PA were partially protected when challenged with Ames strain spores (9, 11, 26). When mice and guinea pigs are immunized with the Sterne strain veterinary spore vaccine (24) or with the *B. subtilis* PA-producing recombinant strains (6, 9, 28, 29), 50 to 70% of mice and 95 to 100% of guinea pigs are protected. However, the Sterne strain spore vaccine kills mice at certain doses (29) and both of these live spore vaccines kill some of the immunized guinea pigs at certain doses (9). In male CBA/J mice, the observed protective efficacy of two new vaccines tested here, baculovirus PA and the live WR-PA vaccinia virus recombinant, was comparable to that of other reported *B. anthracis* vaccines tested in female CBA/J mice, with the exception of the Aro$^-$ *B. anthracis* FD11 experimental vaccine (9). Only Aro$^-$ *B. anthracis* FD11 completely protects female CBA/J mice (9). Baculovirus-PA plus TriMix completely protected guinea pigs against an Ames spore challenge, was superior to the human vaccine MDPH-PA, was as efficacious as the live Sterne spore vaccine, and was superior to the live *B. subtilis* vaccine. In addition, baculovirus PA was not lethal to animals upon immunization.

The results of the studies reported here and elsewhere (3) on the characterization and testing of new virus recombinant PA vaccines in mouse and guinea pig animal models suggest that these recombinant viruses or their products should be further investigated as novel *B. anthracis* vaccines.

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